Influence of hESC derivation conditions on germ cell differentiation potential

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Introduction: Human embryonic stem cells (hESC) have been shown to be more similar to 'primed' mouse epiblast stem cells (mEpiSCs) in contrast to 'naive' mouse embryonic stem cells. Primed EpiSCs, which are dependent on activin signalling, are considered to be poised for germ cell differentiation in response to BMP4 signalling. Correspondingly, *in vivo* primordial germ cell (PGC) specification occurs in the early stages of embryogenesis in the epiblast via BMP signalling. Downregulation of fragilis and appearance of VASA indicates the transition of cells from early to late PGC state. Hence, we hypothesized that hESCs derived in different environmental conditions can affect differentiation towards PGC fate in the presence of BMP4.

Materials and Methods: hESC lines A, B, C and D were derived on MEFs in hypoxic conditions using standard hESC culture medium with bFGF. Only for hESC line A, additional supplementation of 20ng/ml Activin A was used from the blastocyst stage onwards until passage 6. Cells were passaged every 5-7 days to keep them in undifferentiated state. For our experiments, hESCs were differentiated as embryoid bodies (EBs) in media composed of Knockout Dulbecco's Modified

Eagle Media, 20% Fetal Bovine Serum, non-essential amino acids, Pennicillin-Streptomycin, L-glutamine, β-mercaptoethanol supplemented with 50ng/ml BMP4. Differentiation was allowed to proceed for 14 days. EBs were harvested at different days (day 3, 7 and 14) of differentiation for quantitative real-time PCR anlaysis of early germ cell marker Fragilis, post-migratory marker VASA, and pluripotency marker Oct4. Normalized expression values were calculated using three housekeeping genes (GAPDH, B2M, RPL13A). Eleven-week fetal testis was used as a positive control and fold change expression of each gene was calculated relative to its normalised gene expression values. Significance was calculated using T-test and Anova of means and standard deviations of fold change expression values. Immunocytochemistry was performed for hESC lines A and B supplemented with BMP4 on day 14 for VASA and Oct4.

Results: Activin A-derived hESC line A showed significant upregulation of VASA compared to hESC lines B, C and D on day 7 ($p=0.006$). The upregulation of Vasa in A corresponded to a significant downregulation in fragilis ($p=0.011$**)**, whereas, for B, C, and D, fragilis was significantly increased from day 3 to day 7 ($p=0.011$). By day 14, fragilis was significantly upregulated in hESC line A ($p=0.001$) and VASA expression disappeared. In contrast, hESC lines B, C and D continued to show a rise in fragilis expression from day 0 to day 14 ($p=0.001$) while VASA expression continued to be lowly expressed. Oct4 was significantly downregulated in all hESC lines from day 0 to day 14 ($p=0.001$). Immunocytochemistry showed specific cytoplasmic staining of VASA for only Activin A-derived hESC line A and Oct4 was absent in both A and B.

Conclusion: Our results indicated increased germ cell differentiation potential by Activin A-derived hESC line A with respect to hESC lines derived without Activin A .The transition from early to late PGC state for A is reflected by the siginificant

downregulation of Fragilis and the upregulation of VASA from day 3 to day 7. Alongside, disappearance of VASA at day 14 and simultaneous rise in fragilis denotes a critical time point for germ cell fate induction. hESC lines B, C and D failed to show early to late PGC transition as fragilis continued to be highly expressed and VASA expression was almost absent. Immunocytochemistry further confirmed VASA expression at protein level for A. Hence, as hESCs derived in Activin A are considered to be more in a primed state, similar to activin-dependent EpiSCs, future research will focus on validating the interaction between activin signalling and germ cell differentiation *in vitro*.